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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

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R C van Dijk

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If no title is shown please refer to the description.
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Method for solution based diagnosis

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Method for solution based diagnosis

This invention provides methods for improved diagnosis of medically relevant conditions by solution based biochemical testing procedures performed in solutions of test samples. The invention provides a method to substitute the cell based morphological information contained within the cytological and/or histological data of the test sample by molecular information obtainable from the solution, wherein the original test sample is dissolved and thus enables for accurate and reproducible assessment of medically relevant diagnosis from dissolved test samples. The method according to the invention comprises the steps of determining the levels of one or more markers associated with the condition to be diagnosed, determining the level of a set of normalization markers suitable to substitute the information related to morphological aspects of the sample, that would have enabled or supported diagnosis in a cell based test system, comparing and/or combining the data concerning the levels of said markers and assessing diagnosis of a medically relevant condition.

The diagnosis of a large number of medically relevant conditions is nowadays performed using molecular markers as tools. The molecular tools are generally used as one aspect in a complex examination, taking into account a series of different parameters characterizing the samples to be examined.

In medically relevant analysis the morphological examination of samples by cytological or histological means is in common use. Such methods based on morphological characterization of cell based samples are applicable for example in analysis of clinical samples such as body fluids, blood, surgical resections, secretions, swabs or lavages.

In screening for cervical cancer for example swabs are used for detection of neoplastic lesions of the cervix uteri. In the screening procedure lesions of different origin have to be distinguished. Causes for lesions may for example be inflammations (due to infectious agents or physical or chemical damage) or preneoplastic and neoplastic changes. In morphological examinations the lesions of different characteristics are sophisticated to distinguish. Thus, for examination of swabs cytologists and pathologists have to be especially trained and even experienced examiners have a high inter- and intra-observer variance in the assessment of a diagnosis based on cytological specimens. In general the result of the examination is based upon the subjective interpretation of diagnostic criteria by the examining pathologist/cytologist. As a result the rate of false positive and false negative results in the screening tests remains unsatisfying high.

Therefore in many cases these cytological or histological examination procedures are supported by the use of molecular markers. Such markers are often used in immuno-histochemical staining reactions, or in the course of in-situ hybridisation reactions. In the prior art combinations of morphological examinations and immuno-histochemical staining reactions based on marker molecules, characteristic for different medically relevant states of tissues or cells, may lead to enhanced results. The morphologic examination remains laborious and time consuming and thus expensive, even when supported by the molecular methods, that make the results more reliable. Additionally the diagnosis on a morphologically cell based level is, even when supported by molecular parameters, subject to individual perception of the morphology by individual examiners. Thus the diagnosis is dependent on the person, that performs the examinations.

Only in very few cases molecular markers may be used as diagnostic tools without further support by cell based morphological examinations. This is especially the case, if markers are to be detected in an environment, where they do only occur under exactly defined conditions. So the methods for diagnosis of conditions on a molecular level only, without the support of cell based information, are restricted to cases, where there are suitable markers, that are non-ambiguously specific for the condition to be characterized. For example detection of viral infections may be carried out in solutions of samples, because the markers characteristic for the presence of viruses in tissues do not occur in unaffected human tissues.

Thus the reproducibility of the results of examination may be enhanced by the use of supporting molecular tools. Yet the problem with the preservation and preparation of the samples may not be overcome by just additionally using molecular markers.

But especially when using molecular tools in cytological or histological examinations strict precautions in preserving the samples have to be taken to prevent artefacts and improper results of the tests. This is in part due to the instability of the cell based morphological information and in part to the instability of the molecular markers to be detected during the tests. If the samples are not prepared, transported or stored in the appropriate manner, the cell based information, or even the molecular information may get lost, or may be altered. So the diagnosis may be impossible, or may be prone to artefacts. For example the interpretation of biopsies or cytological preparations is frequently made difficult or impossible by damaged (physically or biochemically) cells. Furthermore regarding tissue samples or biopsies the preservation of molecular constituents of the samples, which are subject to a rapid turnover, seems sophisticated due to the time passing by until penetration of the total sample by appropriate preservatives.

In contrast there is a number of robust, fast and easy ways to preserve molecular properties of samples, whereby the morphological information of samples is lost. Samples may be e.g. prepared in a reproducible and easy to store and to transport form by dissolving the cellular components of the raw sample in a suitable solvent immediately after or even during obtaining the sample. Body fluids may directly be transferred from the body of an individual to a solution containing suitable detergents and preservative substances. Furthermore tissue samples may immediately be transferred to denaturing lysis conditions (eventually supported by physical forces) and be thus preserved. Using appropriate ingredients in the solvent the molecular components of the original sample may be preserved, and no degradation may occur. The degradation by enzymatic activities may for example be minimized by the use of enzyme inhibitors. Thus a solution of test samples may easily represent the molecular properties of a test sample at the time of dissolution, without requiring additional preservative precautions.

Due to the dissolving of the sample all morphological, cell based information characterizing the sample are lost. So diagnosis of conditions may not be founded on these data.

The morphologically supported diagnostic methods performed routinely in the art show two major disadvantages. Firstly the methods are highly dependent on individual perception of the examiners. Secondly the morphological information is quite sensitive to decay processes and thus to production of artefacts after preparation of the samples. Both aspects contribute to improper reproducibility of the results.

For improved diagnosis of medically relevant conditions, methods that do not depend on cell based morphological information would be desirable.

A method for performing diagnosis of medically relevant conditions on the basis of molecular markers, characterizing the sample, without relying on morphological cell based information is provided by the present invention.

The present invention is based on the inventors findings illustrated in the Examples 1-4, that diagnosis of conditions, that is normally (in cell based diagnostic systems) enabled and/or supported by histological and/or cytological examination procedures, may be performed in solutions from raw samples containing various cell types of different characteristics, by a method comprising the steps of obtaining a raw sample, dissolving the sample in an appropriate solute, detecting the level of one or more markers associated with the condition to be diagnosed and additionally one or more normalization markers within the sample solution, normalizing the data correlating to the markers

associated with said condition with respect to the data correlating to the normalization markers and diagnosing the presence or absence of a condition in the sample.

The method according to the present invention may for example be applied as a primary screening test in cases, where a cytological, histological or pathological examination is normally performed. Using the present invention one may discriminate, if the condition to be diagnosed may be present in the sample. If the solution based diagnosis gives a negative result concerning a particular condition maybe further examination is omissible. In case of positive results ascertaining by classically applicable methods may follow. Thus expensive and time consuming microscopic or other examinations could be avoided by means of an inexpensive rapid primary screening test.

One aspect of the present invention is a method for enhanced diagnosis medically relevant conditions, wherein the assessment of diagnosis is performed using solutions of lysed raw tissue- or cell-samples. The method for diagnosis disclosed according to the present invention does not rely on morphological parameters but enables for a diagnosis by means of biochemical analysis.

A second aspect of the present invention is a method for characterizing a complex sample in solution by means of molecular markers characteristic for the parameters of interest, thus substituting information, which could otherwise be obtainable from cytological or histological examinations.

A third aspect of the present invention is to provide suitable combinations of markers for the diagnosis of particular conditions of medical relevance in complex samples. The markers for normalization are chosen such that parameters included within the raw sample, that enable or support the diagnosis, which are lost by the dissolution of the sample, may be substituted.

A fourth aspect of the present invention are test kits for performing diagnostic or research studies according to the present invention.

The present invention enables for a rapid and easy assay for diagnosing of conditions in raw samples such as body fluids, swabs, lavages, needle-aspirates or complex cell- or tissue samples. In general a problem with raw materials is the presence of a number of different cell-types within the sample and additionally the presence of particular microorganisms and extracellular substances. Thus the raw material contains a mixture of cells and compositions, that is prone to give artefacts as results. The presence of different cell types with different proliferative characteristics, of organisms and substances within the raw sample gives rise to multiple factors, that may contribute to the particular level of a marker molecule. Detecting solely the level of one single molecular marker may thus only lead to a diagnostically useful information, if further (morphological) parameters concerning the raw sample are provided. All morphologic data obtainable from the raw sample are lost due to

lysis in solution. Yet there are suitable molecular markers corresponding to particular morphologic or other parameters obtainable by histological, cytological methods.

For example the information about the single constituents within the raw sample may be classically obtained by microscopic examination. Morphologic inspection gives hints about the differentiation, the localization of cells, as well as about the environment, in which the cells appear. In cytological preparations of cervical-swabs for example the particular cells may be identified as epithelial cells and further categorized as e.g. endocervical or ectocervical epithelial cells. Even the presence of non-cervical cells such as endometrial cells may be ascertained easily by microscopic inspection.

According to the present invention raw materials may directly be dissolved in an appropriate solvent without further preparation or characterization independent of the homogeneous or heterogeneous character of the sample material. Data, which are lost through lysis of the material are contained within the sample solution encoded by the levels of a series of marker molecules and may thus be reconstructed using said molecular data for normalization to the respective morphologic characteristics. This is achieved by employing a suitable set of molecular markers for each of the characteristic parameters needed for unambiguous diagnosis. By detecting a suitable array of markers one may assess the relevant parameters characterizing the raw sample and thus overcome the disadvantage of loss of information through lysis of the sample.

The testing procedure according to the present invention includes detecting the levels of markers characteristic for cell conditions in question and of markers for normalizing the data with respect to parameters characterizing the particular environment in the test sample. The markers suitable for the present invention may be of various origin. The expression pattern of a marker, that is suitable for the detection of conditions in question, may be dependent on the proliferative status of cells, on the differentiation status, on the cell type or on the organism. Examples for appropriate markers are set forth below.

Diagnosis of medically relevant conditions as used herein may comprise examination of any condition, that is detectable on a cytological, histological, biochemical or molecular biological level, that may be useful in respect to the human health and/or body. Such examinations may comprise e.g. medically diagnostic methods and research studies in life sciences. In one embodiment of the invention the method is used for diagnosis of medically relevant conditions such as e.g. diseases. Such diseases may for example comprise disorders characterized by non-wild type proliferation of cells or tissues.

In one embodiment the diagnosis pertains to diagnosis of cancers and their precursory stages, to monitoring of the disease course in cancers, to assessment of prognosis in cancers and to detection of disseminated tumor cells e.g. in the course of minimal residual disease diagnosis. The method according to the present invention may for example be used in the course of clinical or pathological diagnosis of cancers and their precursory stages or in routine screening tests as performed for particular cancers such as for example for examination of swabs e.g. in screening tests for cervical lesions, of bronchial lavages for lung cancer or of stool for lesions of the gastrointestinal tract, e.g. colorectal lesions.

The method according to the present invention is applicable to all kinds of medically relevant conditions.

Medically relevant conditions as used according to the present invention may for example be compositions of tissues, body fluids, secretions, washes or swabs. Such conditions may for example comprise the cellular composition of body fluids, such as the composition of blood, the composition of liquor or the composition of semen. In this context the compositions shall be for example the presence or absence of particular cell types (e.g. pathogens, such as, viruses etc., preneoplastic, neoplastic and/or dysplastic cells etc.), the presence or absence of differentiation patterns of particular cell types, the total number of a particular cell types (e.g. erythrocytes, leucocytes, sperm, etc.), the total number of all cells of any cell types or the fraction of cells of particular other characteristics present or absent in the sample.

Furthermore medically relevant conditions may also comprise disorders related to cells, or tissues. The conditions to be diagnosed may comprise parameters related to cells in cytological or histological tissue samples. The conditions may comprise a differentiation pattern of cells in a tissue sample, such as surgical resection samples, biopsies, swabs, lavages etc. Such conditions may comprise e.g. congenital disorders, inflammatory disorders, mechanical disorders, traumatic disorders, vascular disorders, degenerative disorders, growth disorders, benign neoplasms, malignant neoplasms. Another aspect of the conditions according to the present invention may comprise conditions characterized by the presence or absence of proliferative characteristics. Conditions characterized by the presence or absence of proliferative characteristics may be for example cell proliferative disorders.

Cell proliferative disorders according to the present invention comprise diseases characterized by abnormal growth properties of cells or tissues compared to the growth properties of normal control cells or tissues. The growth of the cells or tissues may be for example abnormally accelerated, decelerated or may be regulated abnormally. Abnormal regulation as used above may comprise any

form of presence or absence of non wild-type responses of the cells or tissues to naturally occurring growth regulating influences. The abnormalities in growth of the cells or tissues may be for example neoplastic or hyperplastic.

In one embodiment the cell proliferative disorders are tumors. Tumors may comprise tumors of the head and the neck, tumors of the respiratory tract, tumors of the gastrointestinal tract, tumors of the urinary system, tumors of the reproductive system, tumors of the endocrine system, tumors of the central and peripheral nervous system, tumors of the skin and its appendages, tumors of the soft tissues and bones, tumors of the lymphopoietic and hematopoietic system etc.. Tumors may comprise for example neoplasms such as benign and malignant tumors, carcinomas, sarcomas, leukemias, lymphomas or dysplasias. In a particular embodiment the tumor is for example cancer of the head and the neck, cancer of the respiratory tract, cancer of the gastrointestinal tract, cancer of the skin and its appendages, cancer of the central and peripheral nervous system, cancer of the urinary system, cancer of the reproductive system, cancer of the endocrine system, cancer of the soft tissues and bone, cancer of the hematopoietic and lymphopoietic system. In one embodiment the method according to the present invention also pertains to the detection of disseminated tumor cells or metastases.

In one embodiment of the invention the carcinoma is e.g. cervical cancer, colon cancer, gastric cancer, breast cancer, bladder cancer, lung cancer etc.

A raw sample according to the method of the present invention may comprise any sample comprising cells or cell debris. The cells may for example be prokaryotic or eukaryotic cells. Furthermore samples may comprise clinical samples, such as e.g. secretions, swabs, lavages, body fluids, blood, urine, semen, stool, bile, liquor, bone marrow, biopsies, cell- and tissue-samples. Biopsies as used in the context of the present invention may comprise e.g. resection samples of tumors, tissue samples prepared by endoscopic means or needle biopsies of organs. Furthermore any sample potentially containing the marker molecules to be detected may be a sample according to the present invention. In one embodiment of the invention the sample comprises cervical swabs, bronchial lavages, stool etc. Raw sample as used in the context of the present invention may comprise fixed or preserved cell or tissue samples. E.g. cells preserved in suitable solutions (alcohols etc.) or fixed tissue samples may be used as raw samples in the methods according to the present invention.

According to the present invention the raw samples are solubilized in a suitable solvent. Such solvents may for example be aqueous solutions of chaotropic agents such as e.g. urea, GuaSCN, Formamid, of detergents such as anionic detergents (e.g. SDS, N-lauryl sarcosine, sodium

deoxycholate, alkyl-aryl sulphonates, long chain (fatty) alcohol sulphates, olefine sulphates and sulphonates, alpha olefine sulphates and sulphonates, sulphated monoglycerides, sulphated ethers, sulphosuccinates, alkane sulphonates, phosphate esters, alkyl isethionates, sucrose esters), cationic detergents (e.g. cetyl trimethylammonium chloride), non-ionic detergents (e.g. Tween 20, Nonidet P-40, Triton X-100, NP-40, Igepal CA-630, N-Octyl-Glucosid) or amphoteric detergents (e.g CHAPS, 3-Dodecyl-dimethylammonio-propane-1-sulfonate, Lauryldimethylamine oxide) and/or of alkali hydroxides such as e.g. NaOH or KOH. The solvent is designed, so that cells, cell debris, nucleic acids, polypeptides, lipids and other biomolecules potentially present in the raw sample are dissolved. The solution for dissolving the raw samples according to the present invention may furthermore comprise one or more agents that prevent the degradation of components within the raw samples. Such components may for example comprise enzyme inhibitors such as proteinase inhibitors, RNAse inhibitors, DNAse inhibitors etc. In one embodiment of the present invention the sample is lysed directly in the form it is obtainable from the test-individuals. In another embodiment of the present invention the sample may be further purified before being lysed. Such purification procedures may for example comprise washing away of contaminants such as mucus or the like, separation or concentration of cellular components, preserving and transporting of the cells. Thus the cellular components of the raw samples are included in a single sample solution.

The preparation of a sample for use in a method as disclosed herein may also comprise several steps of further preparations of the sample, such as separation of insoluble components, isolation of polypeptides or nucleic acids, preparation of solid phase fixed peptides or nucleic acids or preparation of beads, membranes or slides to which the molecules to be determined are coupled covalently or non-covalently.

According to the present invention the detection of the marker molecules is performed directly from this solution. The detection may be carried out in solution or using reagents fixed to a solid phase. The detection of one or more molecular markers may be performed in a single reaction mixture or in two or more separate reaction mixtures. The detection reactions for several marker molecules may for example be performed simultaneously in multi-well reaction vessels. The markers characteristic for the cell proliferative disorders may be detected using reagents that specifically recognise these molecules. Simultaneously the normalization markers may be detected using reagents, that specifically recognize them. The detection reaction for each class of markers may comprise one or more further reactions with detecting agents either recognizing the initial marker molecules or preferably recognizing the prior molecules (e.g. primary antibodies) used to recognize the initial

markers. The detection reaction further may comprise a reporter reaction indicating the level of the markers characteristic for cell proliferative disorders or the normalization markers.

A probe for the detection of the marker molecules as used in the context of the present invention shall be any molecule, that specifically binds to said marker molecules. The probe may for example be an antigen binding agent such as antibodies (monoclonal or polyclonal), antibody fragments or artificial molecules comprising antigen binding epitopes, DNA or RNA binding molecules such as proteins or nucleic acids. Nucleic acids binding to other nucleic acids may for example be oligonucleotides for detection purposes or primers.

A molecule is said to recognize another molecules if it specifically interacts with that molecule. Specific interaction may for example be specific binding to or of the other molecule.

The reporter reaction may be for example a reaction producing a coloured compound. In one embodiment of the present invention the reporter substances correlated to the particular markers develop different colours. In another embodiment, the normalization marker specific reporter may be a molecule quenching the signal produced by the reporter molecule specific for the marker, characteristic for the medically relevant condition, in dependence on the level of the normalization marker present in the sample. In yet another embodiment the reporter reactions may produce fluorescent dyes with differing wavelength characteristics. In a further embodiment of the present invention the reporter reaction may comprise light emitting reactions with different wavelength characteristics for the reporter substances specific for either marker to be detected. In another embodiment of the present invention the reporter reaction may comprise the emission of radioactive radiation and additional methods for visualizing or quantifying the radiation. In one embodiment, the different marker molecules may be recognized by agents, that bear radio-nuclides emitting radiation with different energetic properties, so that the signals referring to marker molecules could be distinguished.

Applicable formats for the detection reaction according to the present invention may be blotting techniques, such as Western-Blot, Southern-blot, Northern-blot. The blotting techniques are known to those of ordinary skill in the art and may be performed for example as electro-blots, semidry-blots, vacuum-blots or dot-blots. Furthermore immunological methods for detection of molecules may be applied, such as for example immunoprecipitation or immunological assays, such as ELISA, RIA, lateral flow assays, immunochromatographic strips, etc..

The method for detection of the level of the marker molecules in one embodiment of the present invention is any method, which is e.g. suited to detect even very small amounts of specific molecules

in biological samples. Furthermore any method for detection of the marker molecules irrespective of the sensitivity may be applied. The detection reaction according to the present invention may comprise for example detection reactions on the level of nucleic acids and/or detection reactions on the level of polypeptides. In one embodiment of the invention the detection of the marker molecules may comprise the detection of particular splicing variants. In another embodiment of the present invention the detection method may comprise the detection of modifications of marker molecules such as phosphorylation or glycosylation etc of polypeptides or the methylation of nucleic acid molecules in samples.

In one embodiment of the invention the detection of the level of marker molecules is carried out by detection of the level of nucleic acids coding for the marker molecules or fragments thereof present in the sample. The means for detection of nucleic acid molecules are known to those skilled in the art. The procedure for the detection of nucleic acids can for example be carried out by a binding reaction of the molecule to be detected to complementary nucleic acid probes, proteins with binding specificity for the nucleic acids or any other entities specifically recognizing and binding to said nucleic acids. This method can be performed as well in vitro as directly in-situ for example in the course of a detecting staining reaction. Another way of detecting the marker molecules in a sample on the level of nucleic acids performed in the method according to the present invention is an amplification reaction of nucleic acids, which can be carried out in a quantitative manner such as for example the polymerase chain reaction. In one embodiment of the present invention e.g. real time RT PCR may be used to quantify the level of marker RNA in samples of cell proliferative disorders.

In another embodiment of the invention the detection of the level of marker molecules is carried out by determining the level of expression of a protein. The determination of the marker molecules on the protein level may for example be carried out in a reaction comprising a binding agent specific for the detection of the marker molecules. These binding agents may comprise for example antibodies and antigen-binding fragments, bifunctional hybrid antibodies, peptidomimetics containing minimal antigen-binding epitopes etc. The binding agents may be used in many different detection techniques for example in western-blot, ELISA, lateral flow assay, latex-agglutination, immunochromatographic strips or immuno-precipitation. Generally binding agent based detection may be carried out as well in vitro as directly in situ for example in the course of an immunocytochemical staining reaction. Any other method suitable for determining the amount of particular polypeptides in solutions of biological samples can be used according to the present invention.

Methods for the detection of the modified states of nucleic acid molecules and/or polypeptides are known to those of ordinary skill in the art.

Methods for detection of methylation of nucleic acids are known to those of skill in the art and may comprise for example methods employing chemical pre-treatment of nucleic acids with e.g. sodium bisulphite, permanganate or hydrazine, and subsequent detection of the modification by means of specific restriction endonucleases or by means of specific probes e.g. in the course of an amplification reaction. The detection of methylation may furthermore be performed using methylation specific restriction endonucleases. Methods for the detection of methylation states in nucleic acids are e.g. disclosed in patent application EP02010272.9, US5856094, WO0031294, US6331393 etc. The cited documents shall be incorporated herein by reference.

Detection of modified states of polypeptides may for example comprise binding agents specifically recognizing modified or unmodified states of polypeptides. Alternatively enzymes such as phosphatases or glycosylases may be used to remove modifications in molecules. The presence or absence of modifications can thus be detected by determination of mass or charge of the molecules by means of electrophoresis, chromatography, mass spectrometry etc. prior and subsequent to the incubation with a respective enzyme.

In a further embodiment of the present invention the detection of a series of marker molecules is carried out on the level of polypeptides and simultaneously the detection of a further series of marker molecules and/or of all or some of the same marker molecules is carried out on the level of nucleic acids.

Markers associated with medically relevant cellular conditions may e.g. be molecules which influence and/or reflect the proliferation and/or differentiation characteristics of cells and/or tissues. Such molecules may comprise for example cell cycle regulatory proteins, proteins associated with the DNA replication, transmembrane proteins, receptor proteins, signal transducing proteins, calcium binding proteins, proteins containing DNA-binding domains, metalloproteinases, kinases, kinase inhibitors, chaperones, embryogenesis proteins, heat shock proteins or enzymes which modify other proteins posttranslationally thus regulating their activity, or nucleic acids coding for the named proteins. Also mRNA coding for the named proteins may be marker molecules useful according to the present invention. In one embodiment the marker associated with the cell proliferative disorder may be for example uniquely expressed in cells affected by the disorder, may be not expressed in said cells or may be overexpressed in said cells.

Marker molecules for use according to the present invention may comprise one or more markers chosen from p13.5, p14, p15, p16, p19, p21, p27, p53, pRb, p14ARF, cyclin A, cyclin B, cyclin E, MDM-2, MCM2, MCM5, MCM6, CDC2, CDC6, Id1, osteopontine, GRP, renal dipeptidase, her2/neu, TGFßII receptor, HPV associated markers e.g. derived from HPV genes L1, L2, E1, E2, E4, E5, E6 or E7, etc. A selection of markers useful in one embodiment of the present invention for the detection of medically relevant conditions is shown below in Table 1.

In one embodiment the marker for a medically relevant condition may be a marker for tumors (tumor markers). The marker molecules characteristic for tumors may e.g. be proteins, that are expressed in a non-wild type manner in tumors compared to normal control tissue. Non-wild type expression as used herein may comprise increased or decreased levels of expression or lack of expression or expression of non-wild type forms of the respective molecules. Expression of non-wild type forms of a protein may comprise expression of mutated forms of proteins, arising by insertion, deletion, substitution, or frameshift mutations or any other known types of mutations in proteins or nucleic acids. In all cases of the expression of non-wild type proteins or non-wild type levels of proteins the proteins, polypeptides or fragments thereof or nucleic acids encoding these proteins or polypeptides or fragments of these nucleic acids may be used as molecular markers associated with tumors and may thus be understood under the term "tumor marker" as used in the context of the present invention. Proteins that show non-wild type expression in association with tumors are disclosed for example in the documents WO9904265A2, WO0149716A2, WO0055633A2 and WO0142792A2, which shall be incorporated by reference herein.

In one embodiment of the invention the marker characteristic for the medically relevant condition may be a cell cycle regulatory protein such as for example a cyclin, a cyclin dependent kinase or a cyclin dependent kinase inhibitor. In a further embodiment of the invention the marker characteristic for the medically relevant condition may be a marker associated with a transient or a persistent viral infection. The viral infection may comprise an infection by a human papilloma virus (HPV) such as high risk or low risk HPV. The high risk HPV may comprise HPV subtypes such as e.g. HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56 and 58. The markers for HPV infection may e.g. comprise HPV expression products of HPV genes L1, L2, E2, E4, E5, E6 or E7. In a third embodiment of the invention a marker characteristic for a viral infection may be used in combination with any other marker for a medically relevant condition such as e.g. in combination with a cell cycle regulatory protein. Combinations of marker molecules, which may be of special interest with respect to HPV association are e.g. disclosed in WO0208764 which document shall be incorporated herein by reference.

In one embodiment cell cycle regulatory proteins for use in combination with HPV markers may for example be chosen from a group comprising pRb, p53, p14 ARF, cyclin dependent kinase inhibitors. In one special embodiment for example $p16^{INK4a}$ may be used in combination with markers for HPV infection (e.g. L1, L2, E2, E4, E5, E6 or E7).

Normalization markers according to the present invention may comprise for example housekeeping genes, such as actin, gapdh, histone proteins, phospholipase, ß2-microglobulin, proteins associated with active cell proliferation such as e.g. Ki67, PCNA or statin, or proteins characteristic for particular cell types such as for example CK20 in epithelial cells or any cell specific cell-surface antigens. In addition carbohydrate structures present on glycoproteins, proteoglycans, Lectin receptors such as the Concanavalin A Receptor, mucins and enzymes which are involved in the biosynthesis of these molecules such as GalNac transferases and Oligosaccharyltransferases might also serve as normalization markers. The type of marker protein has to be chosen according to the information, which shall be provided by the marker. Principally the markers useful for particular medically relevant conditions may under certain circumstances be useful as normalization markers. A selection of markers useful in performing the methods according to the present invention are given in Table 1.

As well concerning markers for medically relevant conditions as well as concerning normalization markers modified states of molecules (such as polypeptides and nucleic acids) may be used as markers in the method according to the present invention. For example phosphorylated, glycosylated or otherwise modified polypeptides or methylated nucleic acids may be addressed as markers in the method according to the present invention.

Normalization as used according to the present invention shall comprise any method suitable for relating the detected levels of markers to parameters valuable for the assessment of the diagnosis. One aspect of this normalization may be a reconstruction of the relevant cytological and histological information contained within the raw sample by means of suitable molecular markers detectable in the sample solutions. Normalization may comprise for example the detection of the total number of cells present in the sample, of the presence or absence of a particular cell types in a sample, of the presence or absence of an organism or of cells of an organism in a sample, of the number of cells of a particular cell type or organism present in the sample, of the proliferative characteristics of cells present in the sample or of the differentiation pattern of the cells present in the sample.

Table 1:

	cell type	antigen	antibody	supplier	literature

cell type	epithelial cells	human epithelial cell surface glycoprotein	HEA125 IgG1 (W, IHC, ICC, IF)	Research Diagnostics Inc.	Kommoss et al. 2000
		Human epithelial proliferation 40 kD protein (from LoVo)	AUA-1 IgG1 (Elisa)	Research Diagnostics Inc.	Gottschalk et al 1992
		Human epithelial antigen (34+39 kD)	Ber-EP4, IgG1 (IHC, Elisa)	Dako	Latza U et al., 1990 43, 213-9
		Human epithelial proliferating antigen (40 kD)	AUA-1 (Elisa, W, IHC)	Research Diagnostics Inc.	Epentetos, A et al., 1982, 2, 1004-6
	endocervix columnar cells	Cytokeratin 18 (45 kD)	RGE 53, IgG1 (W, IHC, IF)	Research Diagnostics Inc.	Smedts F et al., 1990, 136, 657-68
		Cytokeratin 18 (45 kD	RCK 106 (W, IHF, IHC)	Research Diagnostics Inc	Smedts F et al., 1990, 136, 657-68
		Cytokeratin 8 (52.5 kD)	CAM 5.2 (W, IHC)	BD PharMingen	Smedts F et al., 1990, 136, 657-68
	Endocervical columnar cells	Mucin Antigens (Tn, STn, MUC1, MUC2	DF3	Centocor	Tashiro et al., 1994
	Endocervic Columnar cells	Concanavalin A receptor			Herckenrode et al., 1988, Koch et al., 1986
	Endocervix	GalNacTransferase Oligosaccharyltransferase			Chilton et l., 1988
	Endocervic/Ectocervix	Lectins (ConA, WGA, PNA, UEA I, DBA, SBA, SNA			Di Loretto et al., 1987 Versura et al., 1988
	ectocervix squamous cells	Plakophilin (80.5 kD	PP1-5C2, IgG1 (W, Elisa, IHC, IF)	Research Diagnostics Inc.	Heid, HW, 1994, 58, 113-31
	endometrial cells	Vimentin	VIM 3B4, IgG1, (W, ELISA, IF, IHC)	Research Diagnostics Inc.	Smedts F et al., 1990, 136, 657-68
	Erythrocytes	Haemoglobin	RDI-CBL63, IgM (RIA,EIA)	Research Diagnostics Inc.	Smith et al., J. Histochem. Cytochem. 1998
inflammation	neutrophilic granulocytes NK-cells Macrophages	CD16(NK, Macro, Gran)	DJ130-c, IgG1 (IHC)	DIANOVA	Grundhoever D and Patterson BK, Cytometry 2001;46:340-344
		CD56(NK)	clone Ki-M6		Hermann et al., J. Clin. Immunol. 1990
		CD68(Macro)	(antiCD68)		Cavayal et al., Eur. J. Immunol. 1998(6)1991-2002 (CD56)
	B-cells	CD19 (CD20)	clone AE 1, FACS	DIANOVA	Harrada et al., Blood 1993;81:2658-63 (CD19) Mason et al., Am J. Pathol1990;136:1215-22(CD20)
	T-cells	CD3 (panT cell) (CD4); (CD8)	clone CRIS-7 (antiCD3); IF, IHC,WB	DIANOVA	Jones et al., J Immunol 1993; 150:5429-35
tumor cells	dysplastic and neoplastic cervical cells	$p16^{INK4a}$	E6H4, D7D7	MTM	Klaes R., et. al. 2001
	different cancer cell types	P53 (mutations)			Mendoza-Rodriguez CA, et al., Rev Invest Clin 2001 May-Jun;53(3):266-73
	adeno-carcinoma cells	CEA			Mistretta et al., Experientia, 1974 Rogers et al., Eur. J. Cancre Clin. Oncol. 1984
	bladder cancer cells	NMP22, BTA			van der Poel HG et al., Curr Opin Urol,11,503-509, 2001
	lung cancer cells	PreproGRP			Hamid et al., Cancer, 63, 266-271, 1989, Pagani et al., Int. J. Cancer 47, 371-375, 1991
proliferation	all proliferating cells	PCNA Ki67	Pc10, IgG2a	Zymed	Waseem NH, Lane DP, J Cell Sci 1990:96:121 (PCNA) Cattoretti et al., J Pathol 1992: 168:357-63(Ki67)
infectious agent	HPV 16	E6	BF 7, IgG1 (IHC and in diagnostic kits for cervical swabs)	Research Diagnostics Inc.	Ifner et al., J. Viriol., 1988
		L1	CamVir-1, IgG2a (IP, W, IF, IHC)	Research Diagnostics Inc.	Browne L et al., 1988, 69, 1263-9
	HPV 18	L1	RDI-HPV18-5A3, IgG1 (W, IHC)	Research Diagnostics Inc.	Ifner et al., J. Virol., 1988
			RDI-HPVX-4C4	Research Diagnostics Inc.	Ifner et al., J. Virol., 1988 Gouillou et al., Am. J. Surg. Pathol., 1991

According to the present invention the normalization may comprise the determination of the presence of a number of (human) cells in question in a sample. This is a crucial aspect of the invention. In particular embodiments false (especially false negative) results of tests can only be avoided, if the testing procedure verifies, that the test sample contains the materials (e.g. cells, tissues organisms etc.), that are necessary for performing the particular test. In various tests this will comprise ensuring, that the sample contains cells. In a wide range of embodiments of the invention the verification of the adequacy of the sample will not just comprise ensuring of the presence of cells, but will include the detection of the presence of cells of a distinct origin or of a special cell type.

Thus normalization may also comprise the determination of cells of particular origin such as e.g. cells from a particular organ or of a particular histological localization such as for example the detection of cells of distinct regions of epithelia, or of cells of connective tissue, cells originating from the basal lamina of a tissue or of cells of a heterologous origin, such as metastatic cells. This may be necessary in particular cases, because there might be cells, that under certain circumstances do express a marker, which might be used for the detection of a medically relevant condition, such as e.g. neoplasia or dysplasia, under certain normal conditions. Normalization as used according to the present invention may comprise the detection of the presence or absence and/or the level of any cell-types, that may possibly contribute to the total level of a particular marker selected to diagnose a medically relevant condition.

In one embodiment the method may be applied for the detection of cancers such as e.g. cervical lesions. Markers and combinations thereof useful for this detection purpose are for example disclosed in WO0208764 and EP1217377, which documents shall be incorporated herein by reference. In this embodiment the test may be performed using any suitable sample of cervical origin. The sample may for example comprise biopsies or microbiopsies of the cervix or swabs taken from the cervical region. Cervical swabs as used herein are samples that may for example be obtained using a suitable device such as a brush, a tampon, a spatula or the like, which is contacted with the uterine cervix during the sampling procedure. The sampling device may be any suitable device, which may be used in conventional testing performed by a physician or a self sampling device.

Promising molecular markers for enhancing the evaluation of cervical swabs are e.g. $p16^{INK4a}$, p14ARF, cyclin E, cyclin A, cyclin B, MN, her2/neu, mdm-2, bcl-2, EGF-Receptor, Markers indicative for Human papilloma virus infection, pRb, p53 etc. which might be used to detect dysplastic and neoplastic cells. Normalization according to the present invention for the purpose of analysis of

cervical swabs may comprise the detection of the presence of human cells at all, the detection of cells of the cervical epithelium, the detection of the presence of endocervical as well as ectocervical cells and the detection of cells of endometrial origin. It is a crucial step to ensure the presence of ecto- and endocervical cells within the sample to ensure, that the specimen was taken at the cervical transformation zone, where most dysplasias and neoplasias arise. If there are no such cells, the sample is not adequate for the testing procedure, for it is prone to give false negative results. As $p16^{INK4a}$ may be expressed in normal endometrial cells normalization of the $p16^{INK4a}$ expression level in regard to the number of endometrial cells might be necessary.

To enable for reliable diagnosis the normalization furthermore may comprise the detection of the presence or absence of the named cellular components within the sample, and additionally the detection of the total level of a particular cell type or of the fraction, that a particular cell type contributes to the total number of cells within the sample.

Thus in one embodiment the detected level of the $p16^{INK4a}$ protein may be normalized to the cytological conditions represented by the particular sample, so that one may state, if the detected level of the $p16^{INK4a}$ protein is indicative for cervical cells overexpressing $p16^{INK4a}$, or if there is an abundant number of endometrial cells present in the sample, thus mimicking the overexpression of $p16^{INK4a}$.

In yet another embodiment of the invention the method disclosed herein may be used for the detection of disorders of the respiratory tract. In the diagnosis of small cell lung cancer detection of neuron specific enolase (NSE) is one of the employed markers. Samples of tumor specimens are yielded by bronchoscopy with collection of cells by means of brushes or bronchoalveolar lavages. Since NSE is also expressed in few normal cells within the lung, the level of NSE expression detected in the dissolved sample has to be set in relation to the normalisation maker (for example actin) for detection of the amount of cells present within the sample.

A third embodiment of the present invention is the detection of lesions of the gastrointestinal tract, e.g. colorectal lesions from stool samples. In this case the origin of indicative nucleic acids and/or polypeptides detectable in stool samples may be crucial for the assessment of diagnosis. According to the present invention it is possible to determine the origin (cell types/organism) of the employed marker molecules. Thus false results based e.g. on the detection of marker molecules originating from foodstuff ingested by individuals rather than from lesion of the mucosa of the gastrointestinal tract may be eliminated. Furthermore artefacts produced by the presence of traces of markers from

the blood circulation, or originating from swallowed sputum etc. may be eliminated using the methods disclosed herein.

Another aspect of the present invention is a testing kit for performing the method according to the present invention. The kit may be for example a diagnostic kit, an analytical kit or a research kit.

A kit according to present invention may comprise:

a) reagents for the detection of the marker molecules

b) the reagents and buffers commonly used for carrying out the detection reaction, such as buffers, detection-markers, carrier substances and others

c) one or more markers and/or samples representative for medically relevant conditions to be diagnosed for carrying out positive and/or control reactions

d) one or more normalization marker samples for carrying out a positive and/or control reaction

The reagent for the detection of the marker molecules may include any agent capable of binding to the marker molecule. Such reagents may include proteins, (poly)peptides, nucleic acids, peptide nucleic acids, glycoproteins, proteoglycans, polysaccharids or lipids.

The markers characteristic for medically relevant conditions and/or normalization marker samples for carrying out positive and/or negative controls may comprise for example nucleic acids in applicable form such as solution or salt, peptides in applicable form, tissue section samples, microorganisms or positive or negative cell-lines.

In one embodiment of the invention the detection of the marker molecules is carried out on the level of polypeptides. In this embodiment the binding agent may be for example an antibody specific for the marker molecules or a fragments thereof. Furthermore binding agents may comprise antigen-binding fragments such as Fab fragments, single chain antibodies, bifunctional hybrid antibodies, peptidomimetics containing minimal antigen-binding epitopes etc. Moreover the binding agent might be a lectin binding to a specific carbohydrate structure on the marker molecule.

In an other embodiment of the test kit the detection of the marker molecules is carried out on the nucleic acid level. In this embodiment of the invention the reagent for the detection may be for example a nucleic acid probe or a primer reverse-complementary to said marker nucleic acid.

Brief description of the drawings:

Figure 1: **Specific immunohistochemical staining of endocervical and ectocervical epithelial cells in cervical sections.** Using an antibody directed against cytokeratin 18 (CK18) in an immunohistochemical staining procedure , a positive reaction can be detected in columnar epithelium of the endocervix (A), whereas the squamous epithelium of the ectocervix shows no specific staining (B). Immunohistochemical staining with an antibody directed against cytokeratin 10/13 (CK 10/13) shows no staining in the columnar epithelium of the endocervix (C), whereas there is a strong staining of the squamous epithelium of the ectocervix (D). So CK18 might be used as a specific marker for the detection of columnar epithelial cells of the endocervix and CK10/13 as a specific marker for squamous epithelial cells of the ectocervix. For experimental details see Example 1.

Figure 2: **Western Blot analysis of solubilized samples from cervical swabs**. The numbers 1 to 4 refer to samples (cervical swabs) obtained from individual patients. Immunoblot detection was performed using specific antibodies directed against cytokeratin 8 (CK8) and specific antibodies directed against $p16^{INK4a}$ (p16). The samples of patient 1, 2, and 3 show no signal for $p16^{INK4a}$. This indicates that no dysplastic cervical cells were present in these samples. The sample of patient 4 shows a strong signal for $p16^{INK4a}$. This indicates that dysplastic cervical cells were present in the sample. The upper bands show the specific signals for cytokeratin 8. In sample 1,3, and 4 cytokeratin 8 can be detected, whereas in for sample 2 no signal can be seen. This indicates that endocervical columnar cells were present in samples 1, 3, and 4, and absent in sample 2. As the presence of endocervical columnar epithelial cells is one of the parameters for the adequacy of cervical swabs, sample 2 is considered inadaequat and no diagnostic conclusions can be drawn from the negative result of the $p16^{INK4a}$ detection. Samples 1, 3, 4 are considered adaequat. So based on the negative signal of sample 1 and 3 for $p16^{INK4a}$ it could be concluded, that these patients had no cervical dysplasia. Sample 4 showed a positive signal for $p16^{INK4a}$, indicating the presence of a dysplastic cervical lesion in this patient. For experimental details see Example 2.

Figure 3: **Western blot and ELISA analysis to demonstrate sample adequacy.** Samples of four patients with high-grade cervical dysplasias (see Diagnosis) were analysed using western blot analysis (upper panel of figure). For the left blot immunoblot detection was performed using antibodies specific for β-actin and $p16^{INK4a}$, for the middle blot antibodies specific for cytokeratin 10/13 and for the right blot antibodies specific for cytokeratin 18 were used. β-

actin, CK18, and CK10/13 were used as markers demonstrating the adequacy of the sample. β-actin indicates the presence of any cells, CK10/13 the presence of ectocervical squamous cells, CK 18 the presence of endocervical columnar cells. For the samples of patient 1 and 2 the immunoblot detections show positive signals for all the applied adequacy markers (CK10/13, CK 18, β-actin) and for the marker ($p16^{INK4a}$) indicative of dysplastic cells. For the samples of patient 3 and 4 the immunoblot detections show only very weak (patient 3) or no (patient 4) signals for the adequacy markers, therefore the negative $p16^{INK4a}$ signal does not support any diagnostic conclusions. For experimental details see Example 3. The lower panel of this figure shows the results of ELISA analysis. Positive signals for the adequacy markers (CK10/13, CK 18) were detected for the sample of patient 1 and 2, whereas for the samples of patients 3 and 4 no signals for CK10/13 and CK 18 was seen. So the ELISA analysis results resemble the Western blot analysis results and the same conclusions can be drawn. For experimental details see Example 3.

The following examples are given for the purpose of illustration only and are not intended to limit the scope of the invention disclosed herein.

Example 1: **Specific immunohistochemical detection of endocervical and ectocervical epithelial cells in cervical sections**

In order to evaluate markers indicating the adequacy of cervical swabs, cervical sections (fixed in 4 % formaldehyde solution and paraffin-embedded) were stained with antibodies directed against Cytokeratin 18 (marker for endocervical columnar epithelia) and Cytokeratin 10/13 (marker for ectocervical squamous epithelia). Figure 1 shows specific staining of endocervical epithelia with anti-Cytokeratin 18 antibody and specific staining of ectocervical epithelia with anti-Cytokeratin 10/13 antibody. The experiment was performed as follows:

Formalin-fixed, paraffin-embedded sections were deparaffinized in xylene bath for 5 min (step was repeated once), excess liquid was tapped off and slides were placed in 95-96% ethanol for 3 (±1) min, in 70% ethanol for 3 (±1) min (step was repeated once) and finally in distilled water for a minimum of 30 sec. For epitope retrieval, slides were placed in a Coplin jar and boiled for 40 min at 95-99°C in 10 mM Citrate buffer pH 6.0. Slides were allowed to cool down for 20 min (±1 min) at RT in this buffer. Slides were covered with Peroxidase-Blocking Reagent (3% H_2O_2; NaN_3 15 mM) and incubated for 5 (±1) min at RT. After 5 min washing in washing buffer, slides were incubated with primary antibodies (CK 10/13: DE-K13, 1:50, DAKO; CK 18: K18.7, 1 µg/ml, dianova) for 30 min.

Thereafter, slides were rinsed with wash buffer and washed in wash buffer for 5 min at RT. Following 30 min incubation with EnVision (ready to use anti-mouse horseradish peroxidase-complex; DAKO), slides were washed 3x5 min and incubated in DAB substrate for 10 min, counterstained with hematoxylin and mounted with Faramount mounting medium.

Example 2: **Western Blot analysis of solubilized samples from cervical swabs**

In order to evaluate, whether western blot analysis of solubilized samples allows to assess diagnosis of cervical lesions, clinical samples with known diagnosis were subjected to an immuno-chemical analysis on the basis of marker molecules after lysis of the sample material.

The clinical material (cervical swabs) samples were analyzed by Standard Western Analysis as follows.

In brief the clinical material was in a first step solubilzed by boiling (5min, 95°C) in Lämmli Protein Sample buffer (100 mM Tris pH.6.8, 2% SDS, 200mM DTT, 0.05% BpB) prior to sonification. In a second step protein samples were resolved on a SDS-PAGE (12% Acrylamide) and subsequently transferred on a nitrocellulose membrane by tank blotting (Towbin et al., 1979, Proc Natl Acad Sci;76:4350-4354). In a further step the membranes were blocked to prevent unspecific antibody binding (10% non fat dry milk in PBS) and subsequently incubated with the specific monoclonal mouse antibody (CK 8: 35βH11, 1:100, DAKO; $p16^{INK4a}$: D7D7, 1:140, mtm laboratories). The binding of the specific antibody was visualized by Horseradish Peroxidase conjugated secondary reagents (binding to the marker specific antibody) catalyzing photon emitting substrates.

Cytokeratin 8 (CK 8) was used as an endocervical cell specific marker, indicating the adequacy of the sample collection in the present experiments. The cyclin dependent kinase inhibitor $p16^{INK4a}$ was used as specific disease related marker.

The results of the present experiment are given in Figure 2. The numbers 1 to 4 refer to samples of individual patients. The parallel cytological analysis of the swabs indicated a normal cellular composition for woman 1 and 3. In women 2 no diagnosis due to sparse cellular material could be obtained. In woman 4 a high grade dysplasia was diagnosed. Note that the upper band (CK 8) refers to the endocervical cell specific normalisation marker Cytokeratin 8, indicating the adequacy of the sample collection. The lower band indicates the specific disease related marker $p16^{INK4a}$. The blot shows for patient 4 a positive signal for $p16^{INK4a}$ consistent with a high grade cervical dysplasia. Samples of patient 1 and 3 show only the CK 8 specific band, indicating proper sample collection, but no disease related marker ($p16^{INK4a}$) consistent with a normal, healthy cervical epithelium. The

sample of patient 2 shows no CK 8 signal, consistent with the low cell number in this sample, so no diagnostic conclusion can be drawn from the negative signal for $p16^{INK4a}$.

Example 3: **Western blot and ELISA analysis to demonstrate sample adequacy**

To evaluate, whether results of solution based analysis differing from diagnosis of samples may be due to inadequacy of sample, Western blot analysis of cervical swabs of four different patients with ascertained diagnosis (high-grade cervical intraepithelial neoplasia according to the cytological diagnosis of Pap IVa and Pap IVb) was performed. Antibody against $p16^{INK4a}$ was used to indicate presence of dysplastic cells, whereas antibodies against CK18 and CK10/13 were used to demonstrate adequacy of the sample. As shown in Figure 3, patient samples 1 and 2 showed Western blot signals for $p16^{INK4a}$ and CK10/13 and CK18 (indicating adequacy of samples). Samples 3 and 4 were negative for $p16^{INK4a}$ bands in Western blot. However, in these cases the β-actin and the two cytokeratin markers showed an extremly weak (patient 3 β-actin) or negative (patient 4 all markers, patient 3 CK markers) signal in the Western blot analysis. So no diagnostic conclusion can be drawn from the negative signal for $p16^{INK4a}$. Western blot analysis was performed as follows: Patient samples were collected with a cervical brush and directly lysed in Laemmli Sample Buffer (2% SDS, 60mM Tris pH.6.8, 0.01%, 100 mM DTT) for 5min at 95°C ($1x10^7$ cells/ml) with subsequent sonification (5x5sec pulses, maximum intensity). Lysates were centrifuged for 12min at 16,600xg) in a microcentrifuge and supernatant was transferred into a new tube. Precast 4-20% linear gradient Acrylamide gels (Criterion System, Bio-Rad) were loaded with 10µl (10^5 cells) of whole cell extracts and proteins were separated at 25mA constant current for 45 min. Proteins were transferred from the gel to Hybond ECL Nitrocellulose membrane (Amersham) by standard tank blotting using the Bio Rad Criterion Blotter (15min at constant 100 Volt and subsequently 45min at constant 50 Volt). Nitrocellulose-membrane was stained for 5min in Ponceau S solution to assure protein transfer. Ponceau S solution was removed by 2x10 min washes in PBS. For immunodetection, blots were blocked over night in blocking buffer (10% milk powder in PBS with 0.1% Tween-20). Primary antibodies were incubated at dilutions according to the manufacturer in blocking buffer for 1 h at RT with agitation (CK18: MAB 3236), 1:1000, CHEMICON; CK 10/13: DE-K13, 1:500, DAKO, $p16^{INK4a}$: D7D7, 1:140, mtm laboratories). After 6 washes for 10 min with PBS/0.1% Tween-20, blots were incubated with rabbit anti mouse-HRP, (DAKO, diluted 1:5,000 in blocking buffer) for 1 h at RT. After 6 washes for 10 min with PBS/0.1% Tween-20, membranes were incubated for 5 min in substrate solution (Super Signal West Femto Maximum Substrate, Pierce), wrapped in a plastic envelope and exposed to an x-ray film for 1-5 min. Finally, x-ray films were developed, fixed, dried and documented with an imaging system (Bio-Rad). The same samples were

used to perform ELISA analysis for $p16^{INK4a}$, CK 10/13, CK18. The detected signals and results were the similar to the Western blot analysis and the same conclusions were drawn.

The ELISA analysis was performed as follows: Flat bottom 96 well plates (MaxiSorb; Nunc) were coated with capture antibody ($p16^{INK4a}$: MTM-E6H4, 2µg/ml in PBS, mtm laboratories; CK10: MS481P1ABX, 2µg/ml, dianova; CK18: K18.7, 2µg/ml, dianova; 50µl/well) over night at 4°C. Plates were washed 6x with PBS/0.1% Tween-20 and blocked with Superblock buffer (Pierce). Solubilized protein extract from cervical swabs were dissolved in incubation buffer (PBS, 3% Superblock, 0.1% Tween20), and added in triplicates to each well. After 1 h incubation at RT, plates were washed 6x with PBS/0.1% Tween-20 and incubated with biotinylated detection antibody ($p16^{INK4a}$: MTM-D7D7 (0.2 µg/ml, mtm laboratories, CK10: MS481-BO, 200 µg/ml, dianova; CK18: MS142-BO, 200 µg/ml, dianova; in incubation buffer) for 1 h at RT. Following 6x washes with PBS/0.1% Tween-20 TMB, 50 µl of Streptavidin-coated Alkaline Phosphatase (1:1000 dilution; Dianova) was added for 30 min. Thereafter, plates were washed 6x with PBS/0.1% Tween-20 and 100 µl of p-nitrophenyl phosphate substrate (PnPP; dissolved in diethanol amine buffer) were added to each well. OD 405 nm (620 nm reference wavelength) was measured with an ELISA reader (Tecan) after 30 min, 1h and 2 hrs. The present example shows, that the sandwich ELISA format exhibits sensitivity, which is suitable for the use in the methods according to the present invention. For use in the method disclosed herein the sandwich ELISA format as described in this example may be applied to multiple marker molecules, such as markers for normalization/adequacy and markers characteristic for medically relevant conditions.

Example 4: **Western blot analysis of different samples of pulmonary origin**

In order to evaluate, whether western blot analysis of solubilized samples allows to assess diagnosis of pulmonary lesions, clinical samples with known diagnosis were solubilized and subjected to an immuno-chemical analysis on the basis of marker and normalization molecules.

The clinical samples (cells collected by brushing or bronchoalveolar lavage) were analyzed by Standard Western Analysis as follows. Cells from bronchoalveolar lavage were pelleted by centrifugation (5 min, 1000 rpm) and the pellet was dissolved in Lämmli Protein Sample buffer (100 mM Tris pH.6.8, 2% SDS, 200mM DTT, 0.05% BpB). Cells obtained by brushing were dissolved directly in Lämmli Protein Sample buffer (100 mM Tris pH.6.8, 2% SDS, 200mM DTT, 0.05% BpB). The material was boiled (5min, 95°C) prior to sonification. In a second step aliquots of the protein samples were resolved in duplicates on a SDS-PAGE (12% Acrylamide) and subsequently transferred on a nitrocellulose membrane by tank blotting (Towbin et al., 1979, Proc Natl Acad

Sci;76:4350-4354). In a further step the membranes were blocked to prevent unspecific antibody binding (10% non fat dry milk in PBS) and subsequently one membrane was incubated with specific monoclonal mouse antibodies against NSE (DAKO Germany, clone BSS/NC/VI-H14, mouse monoclonal, dilution 1:1000;) and one membrane was incubated with the normalization marker actin (ICN, USA, clone C4, mouse monoclonal, dilution 1:400). The binding of the specific antibody was visualized by Horseradish Peroxidase conjugated secondary reagents (binding to the marker specific antibody) catalyzing photon emitting substrates.

In the bronchoalveolar lavages of patients with known small cell lung cancers high levels of NSE in comparison with the expression levels of actin was detected, whereas in patients without tumor hardly any NSE could be detected, the actin level however was comparable to the level of the cancer patients. (Data not shown)

The results indicate, that a normalization of the solution based testing procedure according to the method presented herein enables for assessing diagnosis of diseases without relying on morphological information

What is claimed is:

1. A method for diagnosis of medically relevant conditions in raw samples comprising

-preparing a sample solution out of a raw sample

-detecting the levels of one or more markers characteristic for said medically relevant condition in said sample solution

-detecting the levels of one or more normalization markers characteristic for at least one of the following parameters:

- the presence or absence of a particular cell type among the cells represented within the sample solution
- the presence or absence of a particular differentiation pattern in the cells represented within the sample solution
- the presence or absence of particular proliferation properties of the cells represented within the sample solution

-comparing the levels of the markers characteristic for said medically relevant condition to the levels of the normalization markers detected within the sample solution, thus normalizing the detected level of the marker characteristic for said medically relevant condition with respect to one or more suitable parameters; and

-diagnosing the medically relevant condition from the normalized levels of said markers characteristic for said medically relevant condition within the sample solution.

2. The method of claim 1, wherein additionally a quantity of cells contributing to one or more levels of detected markers represented within the sample solution is determined on the molecular marker level.

3. A method according to any one of the claims 1-2, wherein the medical relevant condition is a condition characterized by a property selected from a group comprising the presence or absence of particular cell types in a sample, the presence or absence of a particular differentiation pattern related to cells within the sample and the presence or absence of proliferative characteristics of cells within the sample.

4. The method according to claim 3, wherein the medical relevant condition is a disease.

5. The method according to claim 4, wherein the disease is a cell proliferative disorder, cancer or a precursory lesion.

6. The method according to claim 5, wherein the cancer is cancer of the head and the neck, cancer of the respiratory tract, cancer of the gastrointestinal tract, cancer of the skin and its appendages, cancer of the central and peripheral nervous system, cancer of the urinary system, cancer of the reproductive system, anogenital cancer, cancer of the endocrine system, cancer of the soft tissues and bone, cancer of the lymphopoietic and hematopoietic system.

7. The method according to any one of the claims 1-6, wherein the raw sample is any sample containing cells or cell debris.

8. The method according to claim 7, wherein the cells are cells of an eukaryotic or prokaryotic organism.

9. The method of claim 8, wherein the sample is selected from a group comprising blood, a secretion, a swab, sputum, saliva, stool, bile, a cell- or tissue-sample, a biopsy or a body fluid.

10. The method of claim 1-9, wherein at least one marker characteristic for medically relevant conditions is selected from a group comprising of cell cycle regulatory proteins, metalloproteinases, transmembrane proteins, calcium binding proteins, growth factors, marker molecules characteristic for viral infections, cell proliferation markers and markers associated with DNA replication or the nucleic acids coding for the respective proteins.

11. The method of claim 1-10, wherein at least one marker is a tumor marker protein or the nucleic acids coding therefore.

12. The method of claim 10, wherein at least one tumor marker is selected from a group comprising p53, pRb, p14ARF, cyclin E, cyclin A, cyclin B, MN, her2/neu, mdm-2, bcl-2, EGF-Receptor, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7, CDC2, CDC6, CDC7 protein kinase, CDC14 protein phosphatase, Dbf4, PCNA, Ki67, KiS1, Id1, osteopontine, GRP, renal dipeptidase, TGFßII receptor and cyclin dependent kinase inhibitors.

13. The method of claim 12, wherein the cyclin-dependent kinase inhibitor is selected from a group comprising p13.5, p14, p15, p16, p19, p21, p27.

14. The method according to claim 10, wherein at least one marker is a viral protein or a viral nucleic acid.

15. The method according to claim 14, wherein at least one viral protein is a HPV protein or a nucleic acid derived from a HPV gene selected from the group comprising HPV L1, HPV L2, HPV E1, HPV E2, HPV E4, HPV E5, HPV E6 and HPV E7.

16. The method of any one of the claims 1-15, wherein at least one normalization marker is selected from a group comprising housekeeping genes, cell surface proteins, receptor proteins, glycoproteins and/or proteoglycans, carbohydrate structures specific for glycoproteins and/or proteoglycans, cell cycle regulatory proteins, metalloproteinases, transmembrane proteins, calcium binding proteins, growth factors, cell differentiation markers and proteins associated with DNA replication.

17. The method of claim 16, wherein at least one normalization marker is an epithelial antigen, a cytokeratin or a CD antigen.

18. The method of claim 16, wherein at least one normalization marker is selected from a group comprising a glycoprotein, a proteoglycan and a carbohydrate structure present on these molecules.

19. The method of claim 16, wherein at least one normalization marker is an enzyme involved in the biosynthesis of glycoproteins and/or proteoglycans.

20. The method of any one of the claims 1-19, wherein the detection of the marker molecules is performed using at least one probe specifically recognizing and binding to said marker molecules.

21. A method according to claim 20, wherein at least one probe is detectably labelled.

22. The method according to claim 21, wherein the label is selected from the group comprising of a radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, or an enzyme, a biologically relevant binding structure such as biotin or digoxygenin.

23. The method of any one of the claims 20-22, wherein at least one probe is a binding agent, specifically binding to a marker polypeptide.

24. The method of claim 23, wherein the binding agent is selected from a group comprising an antibody, a miniantibody and a peptidomimetic comprising an antigen binding epitope.

25. The method of any one of the claims 20-22, wherein at least one probe is a lectin comprising a carbohydrate binding site or a carbohydrate specifically recognized by a lectin.

26. The method according to any one of the claims 20-22, wherein at least one probe is a nucleic acid molecule complementary or reverse-complementary to a marker nucleic acid specifically hybridising to said marker nucleic acid.

27. The method of claim 26, wherein the detection comprises a quantitative or semi-quantitative amplification reaction.

28. A method according to any one of the preceding claims, for use in early detection or primary screening tests of cervical lesions, wherein the samples are derived from cervical swab samples.

29. A method according to claim 28, wherein the normalization comprises evaluating on a molecular level whether the sample has been taken in an adequate manner for the purpose of screening for cervical lesions, wherein the detection of markers specifically indicating the presence of endocervical cells is proof for the adequacy of the sample.

30. The method according to claim 29, wherein at least one normalization marker is chosen from a group comprising CK8, CK18, CK10/13, vimentin, concanavalin A receptor and lectins, and wherein at least one marker for the medically relevant condition is chosen from a group comprising an HPV associated marker, p16, p19, p21, p27, pRb, p53, p14ARF, cyclin E, cyclin A, cyclin B, MN, her2/neu, mdm-2, bcl-2, EGF-Receptor, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7, CDC2, CDC6, CDC7 protein kinase, CDC14 protein phosphatase, Dbf4, PCNA, Ki67, KiS1, Id1, osteopontine, GRP, renal dipeptidase, and TGFßII receptor.

31. A test kit for carrying out the method according to any one of the claims 1-30 comprising

-reagents for the detection of two or more marker molecules

-the reagents and buffers commonly used for carrying out the detection reaction, such as buffers, detection-markers, carrier substances and others

-two or more samples of marker molecules for carrying out positive and/or negative control reactions.

32. A test kit according to claim 31, wherein the reagents for detection of marker molecules comprise binding agents specific for said marker molecules and/or nucleic acid probes hybridising to nucleic acids coding for said marker molecules.

33. The test kit according to claim 32, wherein at least one binding agent is an antibody, a miniantibody or a peptidomimetic comprising an antigen binding epitope.

34. The test kit of any one of the claims 31-33, which is a kit selected from a group comprising a diagnostic test kit, a research kit and an analytical kit.

Summary of the invention

This invention provides methods for improved diagnosis of medically relevant conditions by solution based biochemical testing procedures performed in solutions of test samples. The invention provides a method to substitute the cell based morphological information contained within the cytological and/or histological data of the test sample by molecular information obtainable from the solution, wherein the original test sample is dissolved and thus enables for accurate and reproducible assessment of medically relevant diagnosis from dissolved test samples. The method according to the invention comprises the steps of determining the levels of one or more markers associated with the condition to be diagnosed, determining the level of a set of normalization markers suitable to substitute the information related to morphological aspects of the sample, that would have enabled or supported diagnosis in a cell based test system, comparing and/or combining the data concerning the levels of said markers and assessing diagnosis of a medically relevant condition.

Drawings:

Figure 1:

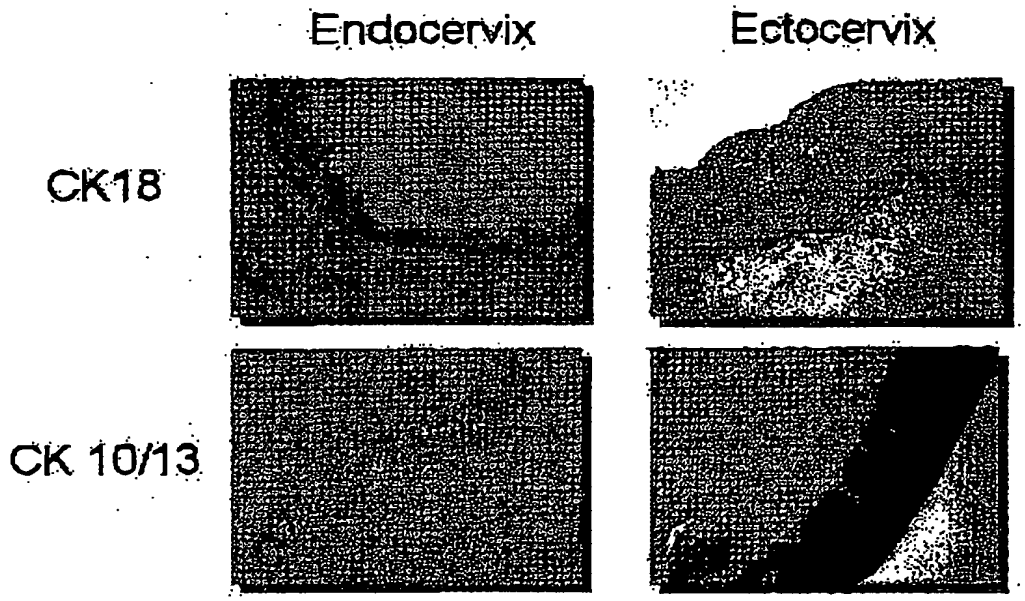


Figure 2:

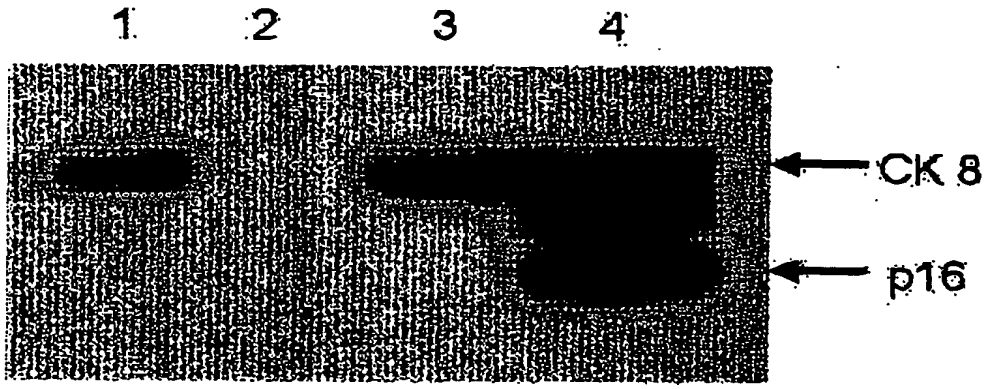
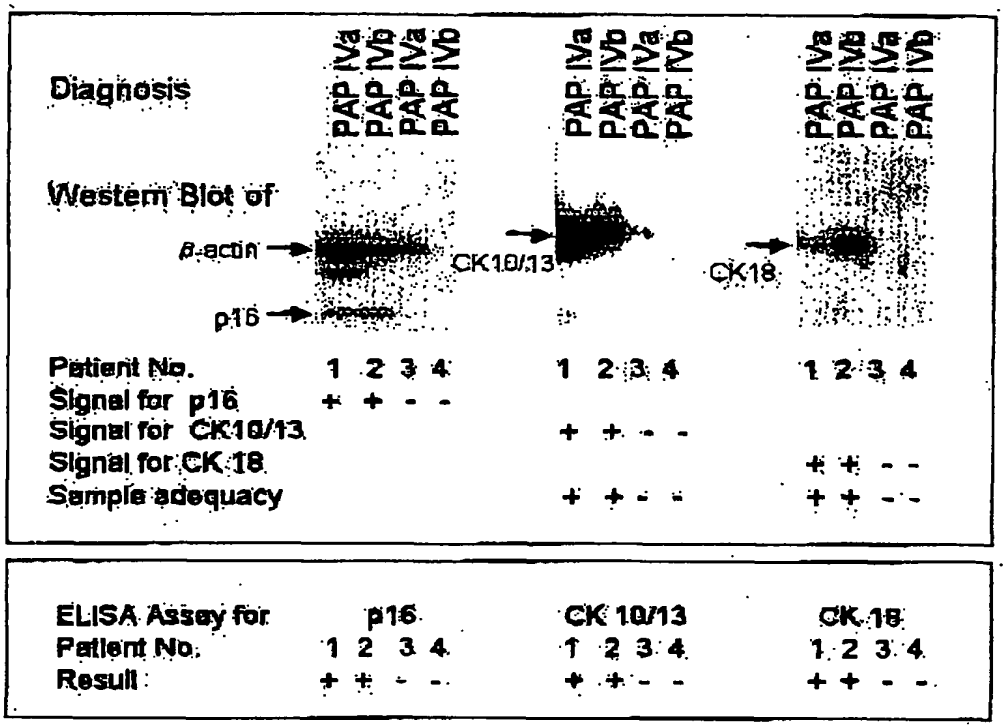


Figure 3:



ELISA Assay for	p16	CK 10/13	CK 18
Patient No.	1 2 3 4	1 2 3 4	1 2 3 4
Result	+ + - -	+ + - -	+ + - -